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Single and joint effects of cadmium and selenium on bioaccumulation, oxidative stress and metabolomic responses in the clam *Scrobicularia plana*

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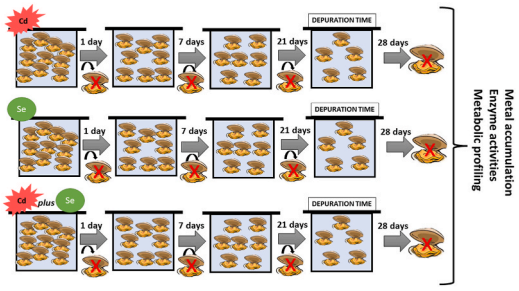
HIGHLIGHTS

- *Clams* accumulated Cd during exposure and reduced its content after depuration.
- Se accumulation was reported only at the highest exposure concentration.
- Se has got an antagonist effect on oxidative stress induced by Cd.
- Cd exposure altered metabolic pathways.
- Se co-administration alleviate the metabolic alterations induced by Cd.

GRAPHICAL ABSTRACT



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ABSTRACT

Selenium (Se) is a vital trace element for many living organisms inclusive of aquatic species. Although the antagonistic action of this element against other pollutants has been previously described for mammals and birds, limited information on the join effects in bivalves is available. To this end, bivalves of the species *Scrobicularia plana* were exposed to Se and Cd individually and jointly. Digestive glands were analysed to determine dose-dependent effects, the potential influence of Se on Cd bioaccumulationas well as the possible recover of the oxidative stress and metabolic alterations induced by Cd. Selenium co-exposure decreased the accumulation of Cd at low concentrations. Cd exposure significantly altered the metabolome of clams such as aminoacyltRNA biosynthesis, glycerophospholipid and amino acid metabolism, while Se co-exposure ameliorated several altered metabolites such asLysoPC (14:0), LysoPE (20:4), LysoPE (22:6), PE (14:0/18:0), PE (20:3/18:4) andpropionyl-L-carnitine.Additionally, Se seems to be able to regulate the redox status of the digestive gland of clams preventing the induction of oxidativedamage in this organ. This study shows the potential Se antagonism against Cd toxicity in *S. plana* and the importance to study join effects of pollutants to understand the mechanism underlined the effects.

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1. Introduction

The occurrence of trace metals in aquatic systems are related to natural and anthropogenic processes being the relative importance of each of them depending on the metal. Among of well-known toxic metals, cadmium (Cd) is of significant concern for human and ecosystem health. The range of its concentration in seawater around the world varies widely between ng L^{-1} in open ocean to few μgL^{-1} in coastal or estuarine ecosystems, but in certain scenarios the concentration can be higher, with values close to 1000 μg L^{-1}. Selenium (Se) is a vital trace element for many aquatic species, although the change to nutrient role as toxic element is very narrow. In fact, it has been recognized as a growing problem of global concern (Chapman, 2009). Se can be mobilized through a wide array of anthropogenic activities. Speciation and biotransformation of Se play important roles in the fate, behavior and effect in the environment. The range of seawater concentration is 0.34–0.50 μg L^{-1} (Kennish, 2000).

The aquatic invertebrates -in general- and more specifically bivalve mollusks have the capacity to accumulate trace metals, as the resulting net process between uptake and excretion. For this reason, they have been employed widely as biomonitoring organisms (Goldberg, 1975; Zhou et al., 2008). However, the accumulation is species and metal specific process because the mechanisms involved in the uptake, excretion and storage are related to adaptation and evolution to ecological changes. The accumulation can be considered as an additive process resulting from dissolved and particulate sources. Cd and Se can be bioaccumulated from water and dietary sources in bivalve mollusks (Wang, 2002). However, the biomagnification process only seems to occur for Se (Barwick and Maher, 2003). This element has been pointed as an antagonist against Cd toxicity, because they form insoluble Cd–Se complexes, Cd also decreases free radicals concentration and increases in the activities of antioxidant enzymes (Rodríguez-Moro et al., 2020).

To avoid the toxic effect, metals and metalloids need to be stored or eliminated to minimize their bioavailability. The mechanisms involved in the toxicity of metals and metalloids can be related to the intracellular formation of reactive oxygen species (Regoli and Giuliani, 2014). For this reason, to assess the effect of metal pollution, the use of biochemical biomarkers as antioxidant enzyme activities and other oxidative stress responses are employed, frequently (Kumar et al., 2021). However, the use of metabolomic approach allow to get insight about the metal-induced changes on metabolic pathways and to decipher the biological responses to this environmental pollution problem. An additional advantage of the use of this approach is the possibility to find new biomarkers in unbiased way to be employed as early warning systems of metal pollution in laboratory and field conditions. These studies have been carried out in tissues and fluids of free-living organisms and *in vivo* specimens in the laboratory (Blasco et al., 2020a, 2020b).

The bivalve mollusk, *Scrobicularia plana* (da Costa, 1778) has been selected for exposure test in this work. This species is recognized as an adequate biomonitor species of environmental quality in aquatic ecosystems (Cajaraville et al., 2000) taking into account its feeding behavior, lifestyle and ecology. This species is widely distributed in the North of Europe, the Mediterranean and West Africa (Santos et al., 2011) and it is key for trophic web in estuarine ecosystems and vector for metal trophic transfer. Due to its human consumption, metal levels can be of concern for human health.

Although, the accumulation and toxicity of Cd and Se have been assessed in species belonging to different trophic levels (Kolarova and Napiórkowski, 2021), it is uncommon to assess the joint action of both elements. The aim of this work has been to analyze the kinetic of accumulation process for both elements as single pollutant and as mixture in a medium-long-term experiment. A recovery period was included at the end of exposure time. Also, to fill the gaps of the information about the response mechanism involved in the individual exposure and to assess the antagonist effect of Se against Cd, a comprehensive approach including metabolomics analyses and traditional enzymatic/non-enzymatic biomarkers was carried out throughout the exposure and depuration phases. We selected biomarkers related to antioxidant and detoxification processes, namely catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST), total-glutathione peroxidase (T-GPx), lipid peroxidation (LPO) and metallothionein (MT, this protein has been widely used as biomarker for metal exposureand oxidative stress due both to its pivotal role in processes of metal handling and detoxification, and to its ability to act as free radical scavenger, (Viarengo et al., 1999; Ruttkay-Nedecky et al., 2013). Additionally, the activity of acetylcholinesterase (AChE) was monitored: this enzyme is traditionally recognized as biomarker of neurotoxicity and its involvement in defense mechanisms against xenobiotics has recently been suggested (Kim and Lee, 2018). This approach can help us to identify affected potential pathways and mechanisms and to discover new biomarkers of exposure and toxicity that can be employed for assessing the effect of these pollutants.

2. Materials and methods

2.1. Experimental design

Bivalves of the species *S. plana* (3.7 ± 0.4 cm shell length) were collected from the Rio San Pedro intertidal mudflat (36°32′00.1″N, 6°12′51.9″W, Cádiz, SW Iberian Peninsula) traditionally considered as a low contaminated area. The animals were acclimated for 7 days in a flow-through seawater (filtered to 0.45 μm) at constant aeration and natural photoperiod.

The exposure experiments were conducted in 62-L tanks (124 organisms/tank, 0.5 L/organims) with prefiltered seawater (0.2 μm), constant aeration, under natural photoperiodand under semi-static conditions. The ratio seawater volume/number of clams in each tank was maintained throughouth the experiment (the volume of seawater was recalculated each time the number of clams changed, both in the case of sampling and removal of dead organisms). The following exposure treatements were included in the bioassay (all in triplicates): seawater control, two different concentrations of dissolved Cd (10 and 100 μg L^{-1}), two different concentrations of dissolved Se (10 and 100 μg L^{-1}), and the mixture of both metals (using the same concentrations of individual assays): 10 μg L^{-1} Cd + 10 μg L^{-1} Se and 100 μg L^{-1} Cd + 100 μg L^{-1} Se). Metals were added to the exposure tanks from stock solutions of 1000 μg L^{-1} (2–3% HNO_3, Merck, Germany). Clams were maintained 21 days under exposure conditions, the seawater was changed every 48 h, and stock solutions were re-added to the water. At the end of exposure period, clams were placed into tanks with clean seawater for 7 days (the total water renewal every 48 h was also maintained in this phase). Both, during the adaptation and experiment period, clams were fed with a commercial filter feeders food (Tropic Marin Pro-Coral Phyton) every 48 h (3 h before water renovation).

Individuals were randomly collected at the start of the experiment (day 0, 78 organisms from the acclimatization tank) and after 1, 7, 21 and 28 days (23 from each tank at each sampling time). The digestive glands were dissected and frozen in liquid nitrogen and stored at −80 °C for chemical, biochemical and metabolomics analyses. Organisms sampled at the days 0 (6 from acclimatization tank) and 28 (5 from each tank) were measured and weighted for the calculation of the Condition Index (CI).

For metal quantification in exposure medium, water samples were collected at the days 0, 2, 16, 18 and 23, acidified with HCl (37%, Suprapur®) 0.1% (v/v) and stored at −20°Cuntil analysis. Conditions of the bioassay were controlled every 48 h (pH 7.9 ± 0.4, temperature 17.8 ± 2.0 °C, dissolved oxygen 7.3 ± 2.1 mg L^{-1}, salinity 35.6 ± 2.2 ppt) and clam mortality daily checked.

2.2. Condition index

The CI was estimated after drying samples at 100 °C during 48 h according to the equation proposed by Lucas and Beninger (1985):

$$dry\ soft\ tissue\ weight\ [g] \times 100 / dry\ shell\ weight\ [g]$$

2.3. Chemical analysis in water and organisms

Water samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Fisher Scientific, Waltham, USA). For quality assurance, run blanks, control and replicates were carried out.

Approximately 0.1–0.2 g of digestive gland (wet tissue) were digested using 2 mL HNO_3 (65%, Suprapur®, Merck, Germany) and 0.5 mL H_2O_2 (30%, Suprapur®, Merck, Germany) with a block digestion system (DigiPREP, SCP SCIENCE) at 95 °C for 1 h and 30 min, and made up to 5 mL with deionized water. Reference material (ERM-CE278k Mussel Tissue certified Reference Material, JRC) were run with each batch. Cd and Se concentrations measured in the reference material were in good agreement with certified values. Instrumental limits of detection (LOD) were determined with the data of calibration curve, 0.1303 and 0.0033 μg L^{-1} for Se and Cd, respectively.

Ammonium levels in water samples were quantified according to the method of Krom (1980) and varied from 26.23 to 130.13 μmol L^{-1}.

2.4. Determination of biomarkers

Digestive gland samples (pools constituted by tissues of 4 individuals, 3 pools per treatment) were homogenized (ULTRA-TURRAX T25, Janke&Kunkel, IKA®Labortechnich) on ice in 50 mM Tris buffer (150 mM NaCl, 1 mM DTT, 0.1% antiproteolitic cocktail at pH 7.4) followed by centrifugation for 30 min (12000×*g* at 4 °C). Supernatants obtained were divided in aliquots and stored at −80 °C for further biomarker determination.

Enzymatic activities and LPO levels quantification were performed by spectrophotometry (Tecan Infinite 200, Tecan Group Ltd, Austria).

CAT activity was determined according to the method described by Li and Schellhorn (2007) measuring the decrease in absorbance at 230 nm due to the H_2O_2 decomposition. SOD activity was assessed using a SOD Assay Kit-WST (Dojindo Laboratories, Japan) according to the method of (Ukeda et al., 1999) SOD from bovine erythrocytes was used as standard.

GPx activity was measured by analyzing (340 nm) the NADPH oxidation in presence of excess glutathione reductase using cumene peroxide as substrate, as described by McFarland et al. (1999).

GST activity was determined (340 nm) using 1-chloro 2, 4 dinitrobenzene (CDNB) as a substrate following the method described by McFarland (1999).

GR activity determination was conducted following the method of (McFarland et al., 1999), monitoring the loss of NADPH present in the reaction mixture at 340 nm.

AChE activity was determined using the method described by (Matozzo et al., 2012) in which reaction progress between acethylthiocholine (ACTC) and 5,5′-dithio-bis (2-nitrobenzoic acid) is followed by recording absorbance (405 nm) changes.

The LPO levels were quantified by measuring (535 nm) malondialdehyde (MDA) concentration, according to the thiobarbituric acid reactive substances (TBARS) method (Hannam et al., 2010).

Total MT content was determined in the supernatant by RP-HPLC coupled to fluorescence detection (W2695 Separation Module, 2475 Multi λ fluorescence Detector, WATERS Corporation, Milford, MA, USA) using rabbit liver MT-I as a reference for standard curve, according to the protocol developed by (Alhama et al., 2006).

All biomarker endpoints were normalized for the total protein concentration of each sample. The protein concentration was determined using the Bradford method (Bradford, 1976), using bovine serum albumine (BSA) as standard.

2.5. Sample preparation for metabolomic analysis

Digestive glands of *S. plana* were individually cryomogenized (SEPX SamplepREP) under liquid nitrogen for a total time of 30s (ten strokes/second).

Metabolites extraction for untargeted metabolomic analysis was developed following a previously reported methodology (Fernández-García et al., 2020) and analysed combining two complementary analytical instrumentation: gas chromatography-mass spectrometry (GC-MS) with ion trap detector (Thermo Fisher Scientific, Germany) and liquid chromatography coupled to a quadrupole time of flight mass spectrometry (UPLC-QTOF-MS). Abbreviated, 30 mg of each individual organ were mixed with precooled methanol (100μL/10 mg) and homogenized for cell disturbance with a pellet mixer (VWR, England, UK) during 2 min. After that, the extracts were centrifuged at 12825 g during 10 min at 4 °C, and then two aliquots were separated from the supernatant: one aliquot of 50 μL was transferred to a vial for GC-MS study and the residual extract for UPLC-QTOF-MS analysis. Extracts were dried with a speed vacuum system and later cold storage at −80 °Cuntil the analysis. For UPLC-QTOF-MS analysis, 100 μL of a mixture containing acetonitrile/water (1:1, v/v) was added to the dried extracts and then injected into the system.

The analysis of the samples by GC-MS involved several previous steps of derivatizationusing the optimized methodology by Begley et al. (2009). To monitor the stability of metabolomics analysis, quality control samples (QCs) were prepared addingidentical volume of individual extracts and analysed following the same procedure as the samples.

2.6. Metabolomic approach by UPLC-ESI-QTOF-MS and GC-MS

Metabolomic experiments by UPLC-ESI (+/−)-QTOF-MS were carried out with an Agilent 6550 iFunnel Q-TOF LC/MS System (Agilent Technologies, Tokyo, Japan) coupled to an Agilent 1290 Series LC pump provided with reversed phase chromatographic column (Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm)). The metabolites were separated following a reported chromatographic condition (Xiong et al., 2018). Two reference masses, for mass correction, were constantly used throughout the analysis: *m/z* 121.0509 and *m/z* 922.0098 for positive ionization mode and *m/z* 112.9856 and *m/z* 1033.9881 for negative ionization mode. The signal was monitored in the range of 50–1100 *m/z*.

The GC-EI-MS analyses were carried out on a Trace GC ULTRA with an ITQ900 ion trap mass spectrometer detector (Thermo Fisher Scientific) equipped a column VF-5MS (30 m × 0.25 μm ID, with 0.25 μm film thickness (Varian)). Samples were injected in splitless mode with an autosampler. The chromatographic condition started at 100 °C during 0.5 min and increased 15 °C min^{-1} until 320 °C, and it was kept for 7 min. The analyses were carried out in full scan mode with the mass range of 40–650 *m/z* and ionization by electronic impact (EI) with a voltage of 70eV. The injector temperature was maintained at 280 °C, and helium was used as carrier gas at 1 mL min^{-1}.

2.7. Data analysis

R package was also used for statistical analysis of bioaccumulation and biomarker data. Statistical significance was established at $p < 0.05$. Bioaccumulation and biochemical data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Leven's test). One and two-way ANOVA were used to study the effects of treatments and multiples comparisons (Bonferroni and T-test) were carried out to evaluate significant differences between treatments.

Mass Profinder version B.10.00 (Agilent Technologies) was used for data pre-processing for the data collected after LC-MS analyses including different molecular features extraction (MFE). After that, data obtained

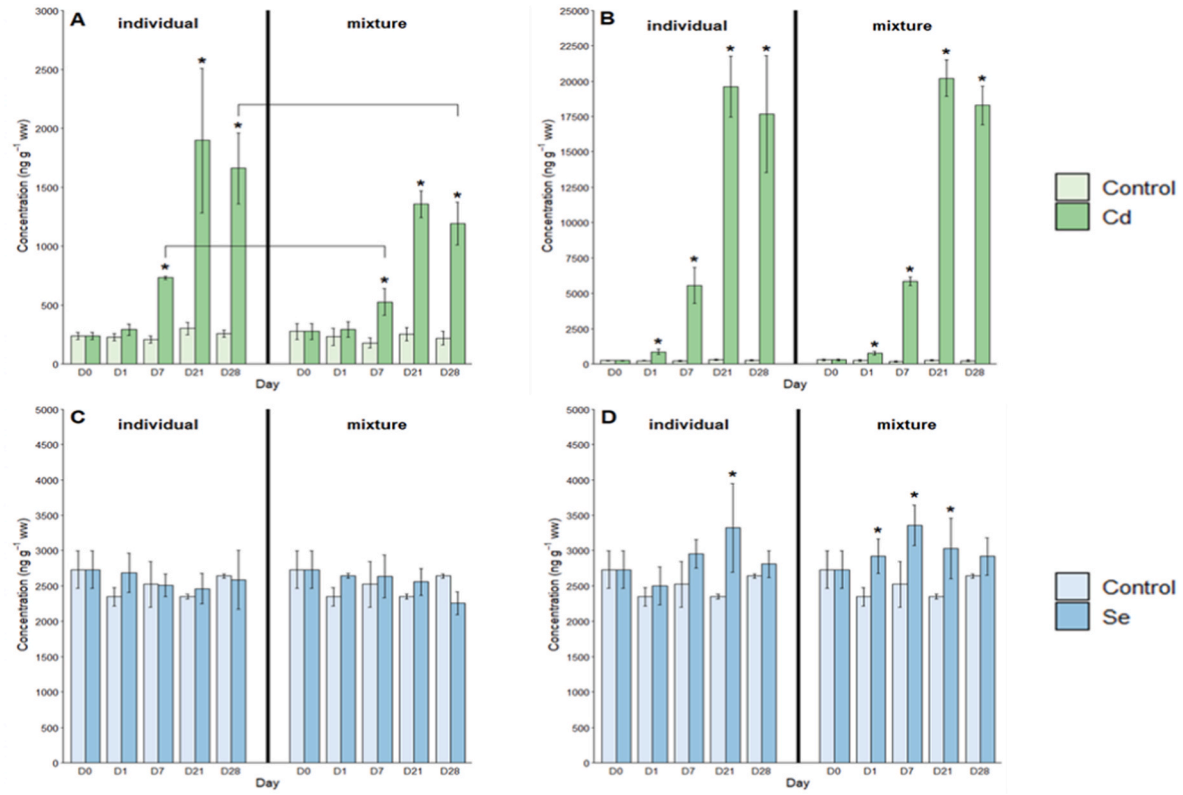

Fig. 1. Bar chart of Cd and Se concentration in digestive gland of *Scrobicularia plana* after Cd and Se exposure at different time.

were imported to Mass Profiler Professional (MPP) B.8.00 using Partial least square discriminant analysis (PLS-DA) to stablish differences among the exposed groups and R^2 and Q^2 parameters was used to evaluate the quality of the built models. Also, statistical analysis, one-way ANOVA ($p < 0.05$) was performed to evaluate differences for each metabolite between exposure groups. Benjamini-Hochberg correction was carried out to test false discovery rate ($\alpha = 0.05$). Finally, the annotation of compounds was carried out by MS/MS experiments and with anidentical resulting fragmentation profile with the available databases METLIN included in the software.

R platform was used for data pre-processing after GC-EI-MS analysis (Katajamaa and Orešič, 2007). SIMCA-P software (version 11.5) was used to PLS-DA analysis and establish differences among exposed groups. Finally, Mass Spectral Library (version 08), included in the software, was used to identify the unknown metabolites (matching spectrum >80%), and Kovat's retention indices, calculated with a n-alkane mix from C10 to C40. Finally, one-way ANOVA and Benjamini-Hochberg correction was carried out following the steps described in the previous section.

3. Results

The average mortality recorded throughout the experiment was 9.3%. No statistical differences were observed between exposed and unexposed organisms with the exception of clams exposed to the mixture of Cd and Se at 100 μg L^{-1} that show significantly lower mortality compared to the control. CI values ranged from 4.5 to 18.7. CI did not change with time and significant differences were not found between experimental groups.

3.1. Metals in exposure water and clams

Water levels of Cd and Se in the controls tanks throughout the entire experiment were respectively 4.8 ± 0.4 and 8.8 ± 0.4 μg L^{-1}. Measured concentrations of Cd and Se in seawater during the exposure phase were at 13.4 ± 0.7 and 18.7 ± 1.9 μg L^{-1}, respectively for the low concentration and at 88.6 ± 8.2 and 110.1 ± 9.1 μg L^{-1}, respectively for the high concentration. Concentrations of Cd and Se measured in the water during the depuration phase were similar in all tanks and in range of values obtained for controls tanks (4.7 ± 0.2 and 9.2 ± 0.3 μg L^{-1} respectively for Cd and Se).

Clams were shown to progressively accumulate Cd in the digestive gland throughout the exposure phase under all exposure conditions (Fig. 1A and B). A tendency to reduce the Cd content was observed when clams are depurated in clean water. When clams were exposed to Se at low concentration (both individually and together with Cd) no accumulation was observed in digestive gland (Fig. 1C). However highest levels with respect to the controls were recorded when clams were exposed to the metal at 100 μg L^{-1} particularly as a mixture with Cd (Fig. 1D). In this last case the levels of Se were reduced after one-week depuration.

3.2. Biochemical responses

The results for the biochemical analysis are shown in Table S1. Cadmium exposure caused the increase of SOD activity in digestive gland in early exposure phase (day 1, 10 and 100 μg L^{-1}, $p < 0.05$). Also AChE activity was significantly increased ($p < 0.05$), but only at 10 μg Cd L^{-1} and at the end of exposure phase. Finally, the GR activity showed a significant enhancement after 7 days of exposure to Cd (100 μg L^{-1}, $p < 0.05$) and subsequent reduction (day 21, $p < 0.05$). After 1 week of depuration we recorded increased levels ($p < 0.05$) of GR (both exposure concentrations) and GST (10 μg L^{-1}) activities, and MT levels (10 μg L^{-1}).

The exposure to Se induced a general decrease ($p < 0.05$) of biochemical parameters in digestive gland of clams: CAT (10 μg L^{-1}, day 1), GPx (10 μg L^{-1}, day 7), GR (10 μg L^{-1}, day 21) GST (10 μg L^{-1} at the day 1, both exposure concentrations at the days 7 and 21) and MT (100

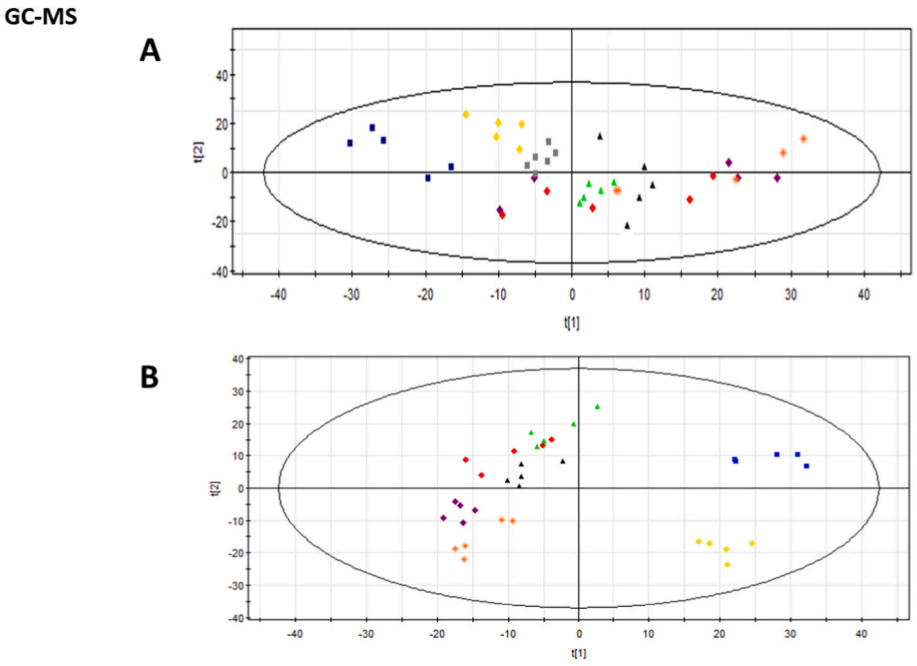

Fig. 2. PCA and PLS-DA score plots (black: control; Se10: red; Se100: green; Cd10: blue; Cd100: yellow; CdS10: purple; CdSe100: orange and QCs: grey) from *Scrobicularia plana* after GC-MS analysis.

μg L^{-1}, day 21). After the depuration phase only a reduction of GR activity ($p < 0.05$) at the high exposure concentration was recorded.

The exposure to the mixture of Cd and Se induced a general enhancement ($p < 0.05$) of responses related to the oxidative stress in digestive gland after 24 h: SOD (100 μg L^{-1}), CAT (10 and 100 μg L^{-1}), GPx (10 μg L^{-1}), GR (10 and 100 μg L^{-1}), GST (10 and 100 μg L^{-1}) and LPO (10 and 100 μg L^{-1}). In the following days of exposure, the enzyme activities and LPO levels were similar or lower than control. Also MT levels decreased significantly after 7 and 21 days of exposure (10 and 100 μg L^{-1}, $p < 0.05$). After one-depuration week biochemical parameters were similar (SOD, CAT, GPx, GST, LPO and MT) or lower (AChE and GR at both exposure concentrations, $p < 0.05$) than control.

3.3. Metabolomic approach by mass spectrometry

To delve into the potential metabolic disorders caused by Cd exposure and the antagonistic role of Se in digestive gland of *S. plana*, two complementary analytical platforms were combined, LC-MS and GC-MS, which allows greater metabolic coverage. The metabolic study was focus on the samples taken at 21 days, which corresponds to the longest period of exposure to these compounds. Fig. 2 shows score plots of PCA and PLS-DA obtained after data processing of the samples analysed by GC-MS. The score plot representing the PCA (Fig. 2A) shows a good cluster of the quality group (QCs), demonstrating the good reproducibility and low variability of the analytical method performed. Fig. S1 (see supplementary information) shows PLS-DA score plots for GC-MS analysis. It can be observed that clams suffered metabolic disorders in digestive gland after Cd and Se exposure, both individually and as mixture, during 21 days and all groups are clearly separated, indicating the presence of discriminative metabolites versus the control group. The annotation of *m/z* variables for each comparison, were identified by MS/MS experiments and applying the statistical analysis described in the experimental section to find the discriminative metabolites in the different exposure groups. Table S2 shows altered metabolites found after UPLC- ESI (+/−)-QTOF-MS and GC-MS analysis. Additionally, Fig. 3 shows a heatmap with these altered metabolites.

Venn diagrams have been drawn (Fig. 4) to find common and different metabolites in clams exposed to Cd versus clams exposed to Cd with Se simultaneously, in order to assess thepotential antagonistic effect of Se against the toxic action of Cd.

Furthermore, for a better understanding, Metaboanalyst 4.0 web tool was employed to find the most significant pathways altered after the exposure to Cd and Se individually and as mixture (Fig. 5). The exposure groups present alterations in Aminoacyl-*t*-RNA biosynthesis and glycerophospholipid metabolism. Moreover, in the groups exposed to Cd, at both 10 and 100 μg L^{-1}, we can observe alterations in other metabolic routes such as arginine biosynthesis or alanine/aspartate/glutamate metabolism (10 μg Cd L^{-1} group) and glyoxylate/dicarboxylate metabolism and alanine, aspartate and glutamate metabolism (100 μg Cd L^{-1}group).

4. Discussion

Since digestive gland of bivalves is the main target organ for heavy metal accumulation (Panfoli et al., 2000), it has been widely used to determine metal bioaccumulation and potential toxic effects in model species. In this work, the dose-dependent and kinetic effects of Cd and the potential antagonistic impact of Se on oxidative stress and the metabolism were studied by biochemical markers and metabolic profiling respectively.

Se remained practically constant in the digestive gland tissues of organisms exposed to 10 μg L^{-1}. Similar results have been observed in the blue mussel *Mytilus edulis* exposed to 4 μg Se L^{-1} for 6 days (Trevisan et al., 2011). These authors argued that, due to the very low exposure concentration, bioaccumulation is undetectable. When Se intake exceeds the amount necessary for homeostasis, it is rapidly excreted and accumulation only occurs if the intake is much higher than the maximal excretion rate. This is consistent with the results obtained in the case of the exposure of *S. plana* to Se 100 μg L^{-1} in which the levels of Se recorded in digestive gland were significantly higher than controls.

Cd showed a continuous accumulation in digestive gland throughout the 21 days of exposure, while a decrease in the concentration of this element was observed after the depuration phase. The digestive gland is an important target tissue for chemical pollutants so it could play an essential role forstoring, metabolizing and processing the degradation/elimination of xenobiotic compounds in bivalves. Interestingly, Fig. 1

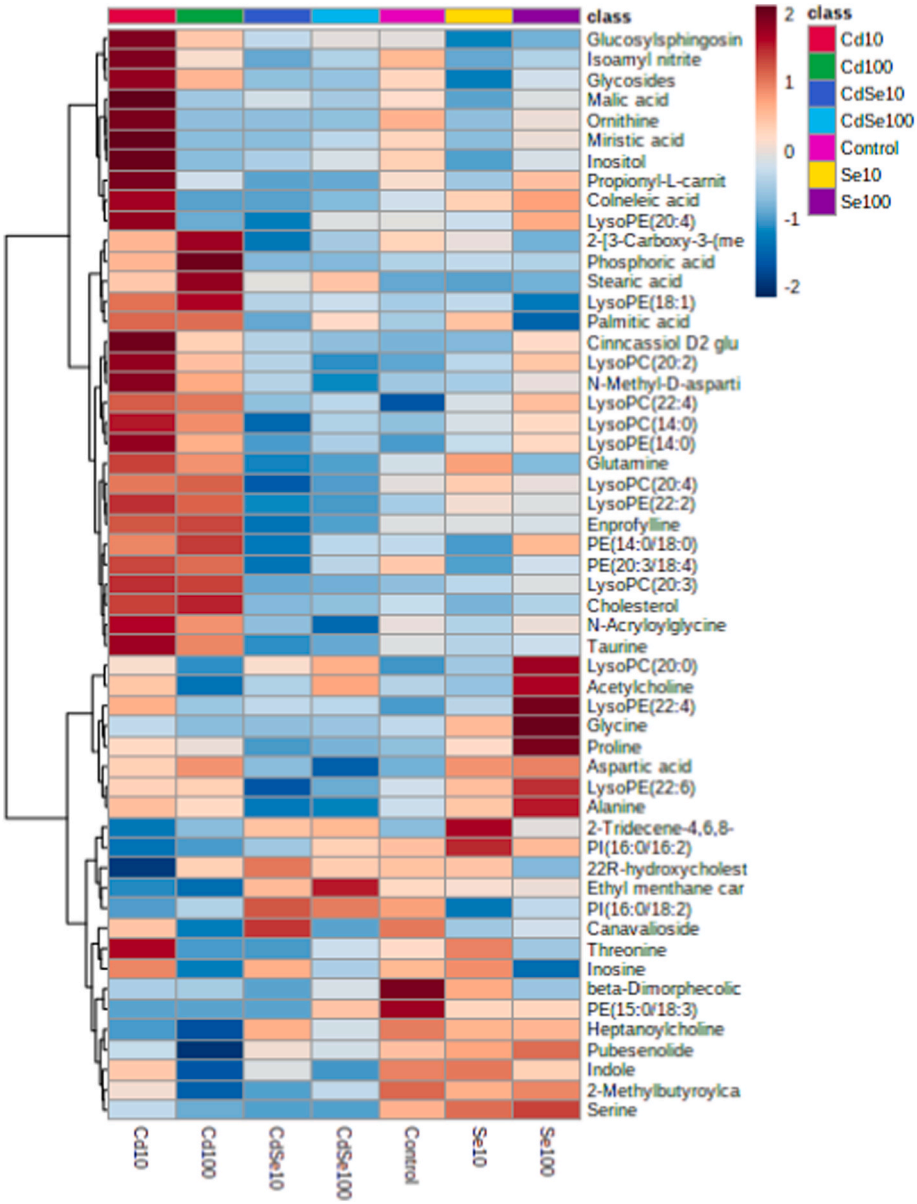

Fig. 3. Heatmap analysis for metabolites identified in digestive gland of *Scrobicularia plana* after 21 days of exposure to Cd and Se.

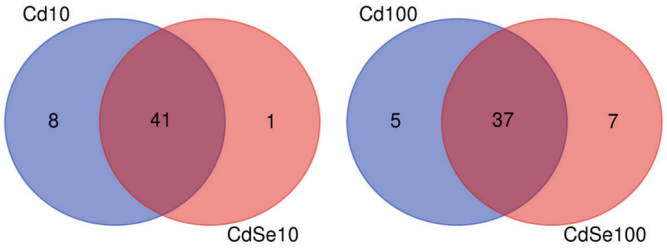

Fig. 4. Venn diagrams showing common and uncommon metabolites altered in digestive gland after Cd and Cd and Se exposure at different concentration (ng g^{-1}): a) 10 of Cd vs 10 of Cd + Se administered simultaneously and; b) 100 of Cd vs 100 of each Cd + Se administered simultaneously.

shows lower accumulation of Cd when the clams are simultaneously exposed to Cd and Se after 7 days of exposure and also after 7 days of depuration in clams exposed to 10 μg L^{-1}. Nevertheless, this trend was not observed in clams exposed to higher concentration (100 μg L^{-1}) of the metals. The studies in aquatic organisms are scarce and have shown different patterns depending on specie and tissue. Xie et al. (2016) observed reduced Cd accumulation in the killifish *Heterandria formosa* pre-exposed to Se. Similarly, a Se-enriched diet decreased the accumulation of Cd in the marine gastropod *Haliotis discus hannai* (Luo et al., 2019). In contrast (Bjerregaard, 1982) observed an increased Cd accumulation in gills and carapace of the crab *Carcinus maenas* in presence of Se in the exposure seawater.

Cd exposure, even at low concentrations, cause a wide variety of adverse effects in aquatic organisms, including metabolism, growth and reproduction alteration, and induction of oxidative stress, genotoxicity, nephrotoxicity and embryotoxicity (Bertin and Averbeck, 2006; Liu et al., 2021; Rani et al., 2014; Zhou et al., 2008) most of them related to an overproduction of reactive oxygen species (ROS) induced by metal.

In this study we recorded an early increase in SOD activity in clams exposed to Cd (both exposure concentrations) suggesting a rapid breakdown of $O_2^{\bullet-}$ radical by the enzyme to keep their level in control and to counteract oxidative stress. The increase of SOD activity as first line of defence against ROS after Cd exposure has also been reported in several aquatic invertebrate such as the clams *Ruditapes philippinarum* and *Ruditapes decussatus,* the freshwater crab *Sinopotamon henanense*,

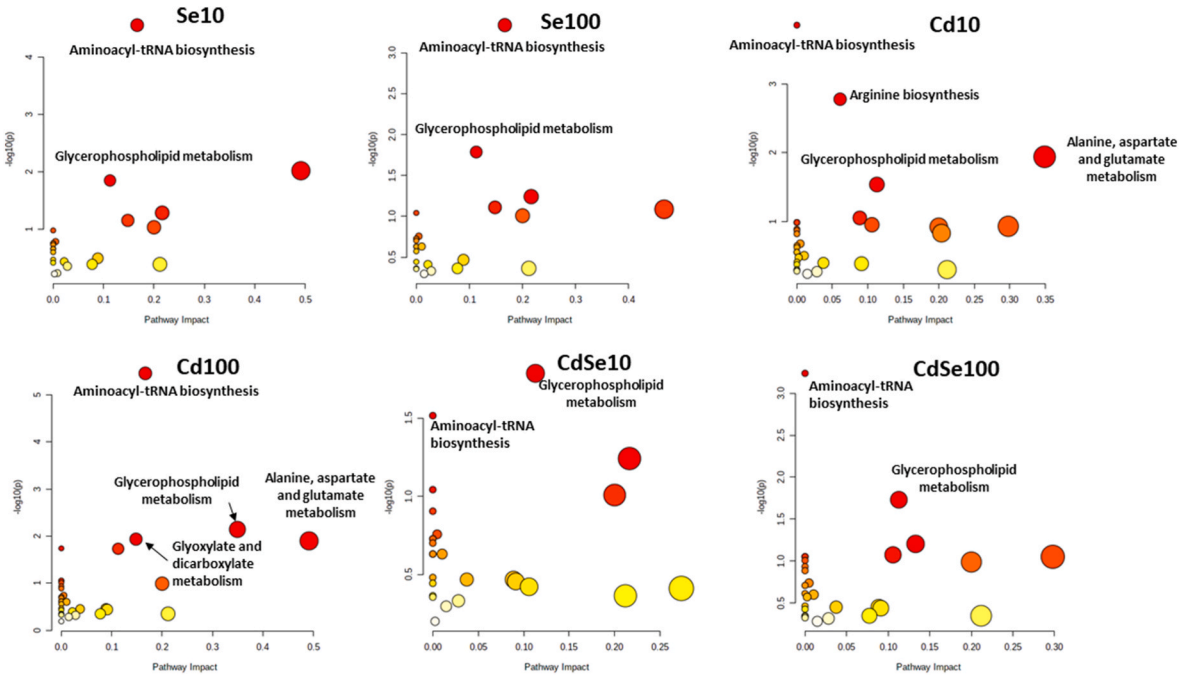

Fig. 5. Altered metabolic pathways in digestive gland of *Scrobicularia plana* after Cd and Se exposure at 21 days.

and in larvae of *Chironomus riparius*, *Chironomuss ancaticaroli* (Figueira et al., 2012; Rebechi et al., 2021; Wang et al., 2011). At the high exposure concentration, changes in the GR activity were also observed; this enzyme, despite not really being an antioxidant enzyme, is essential in maintaining the correct ratio between reduced and oxidized glutathione (GSH/GSSG) and the intracellular redox status in the organism (Regoli and Giuliani, 2014). The efficiency of the antioxidant defence system is confirmed by the absence of damage to the lipid membranes (no increase in LPO levels). The antioxidant system was still active even after one-depuration week suggesting that metabolic processes probably related with metal depuration (GST and MT) and redox balance maintenance (GR) are ongoing. Inhibition of AChE activity is a well-established biomarker of neurotoxic processes (Bocquené and Galgani, 1998; Van derOost et al., 2003; Vieira et al., 2008). In our study, we observed a tendency towards an increase in AChE activity under Cd exposure (although significant only at 10 μg L^{-1} on day 21). In a previous study we observed that in *S. plana* (digestive gland and gills) the AChE activity also increases as a consequence of exposure to a pharmaceutical mixture (Trombini et al., 2022). Enhancement of AChE activity has also been reported in aquatic organisms in response to exposure to metals such as aluminium (muscle and brain of the zebrafish *Danio rerio*), lead and cadmium (digestive gland of the mussel *Perna*) and can be explained as an alteration of the normal function of the enzyme as a consequence of its interaction with metals (changes in conformational state, interaction with acetylcholine receptors) and the increase in the intracellular ROS content (Bainy et al., 2006; Ferrandino et al., 2022; Senger et al., 2011). In addition, we can suggest that the enhancement of AChE activity can be related to the existence for this enzyme of functions other than neurotransmission, such as the involvement in defence mechanisms against xenobiotics (Kim and Lee, 2018).

Se plays significant role in defense of antioxidants and immune functions; both in plants and mammals it is proven that, at low concentrations, this element participates in the preservation of cellular redox balance and in the improvement of the defense system by detoxification of ROS and regulation of enzymatic and non-enzymatic molecules (Abo El-Magd et al., 2022; Chen et al., 2020; Lanza and Reis, 2021; Silva et al., 2020). In our study, no oxidative damage was recorded in organisms exposed to Se concentrations. Additionally, a general decrease in the activity of most of antioxidant enzymes was observed. In this case, Se, thanks to its anti-oxidative and anti-inflammatory properties, could regulate the redox status of digestive gland of clams, keeping it at an even lower level than controls.

An important consequence of the Se properties is the antagonistic effect of this element versus a wide range of compounds, particularly heavy metals. A considerable number of studies carried out on human, vertebrate and plants have shown that Se administration protects against Hg, As and Cd toxicity by reduction of metal-induced oxidative stress and enhancement of the antioxidant capacity of the exposed organisms (Branca et al., 2018; Feng et al., 2013; Ge et al., 2021; Messarah et al., 2012; Sharma et al., 2017). Zhang et al. (2020) observed that Se alleviates the effects of Cd exposure (oxidative stress, apoptosis and necrosis induction) in the lymphocytes of the common carp *Cyprinus carp*. Beneficial effects of Se versus Cd toxicity were also observed in zebrafish and in the rainbow trout *Oncorhynchus mykiss* (reduction of ROS production and LPO level, prevention of antioxidant enzyme inhibition), in the killifish *H. formosa* (reduced levels of lipid peroxidation and increase in antioxidant activity), and in the abalone *H. discus hannai* (enhancement of MT generation and GPx enzymatic activity) (Banni et al., 2011; Luo et al., 2019; Orun et al., 2008; Xie et al., 2016).

As a consequence of the exposure of *S. plana* to the mixture of Cd and Se (both exposure concentrations), we observed an early activation (day 1) of antioxidant defense system and the increase of MDA levels indicative of damage to the lipidic membranes. The decrease of antioxidant responses and the absence of oxidative damage over the following days of exposure indicate the recovery of the redox balance supporting the theory of the protective role of Se against Cd toxicity. Additionally, it seems that the protective effect of Se is also maintained throughout the depuration phase at the end of which reduced activity of several enzymes was observed.

Se can alleviate Cd toxicity via different ways. First, directly regulating the Cd uptake and bioavailability, binding the metal to selenoproteins (Feng et al., 2013; Luo et al., 2019; Xie et al., 2016). Secondly, detoxifying intracellular ROS by the increase of enzymatic activity; Se is a co-factor required for the activity of numerous selenoenzymes involved in the maintenance of redox homeostasis and stress responses among them thioredoxin reductase, iodothyroninedeiodinase and various enzymes of the GPx family (Flora and Mittal, 2015; Ge et al., 2021). Therefore, the joint action of selenoenzymes and antioxidant

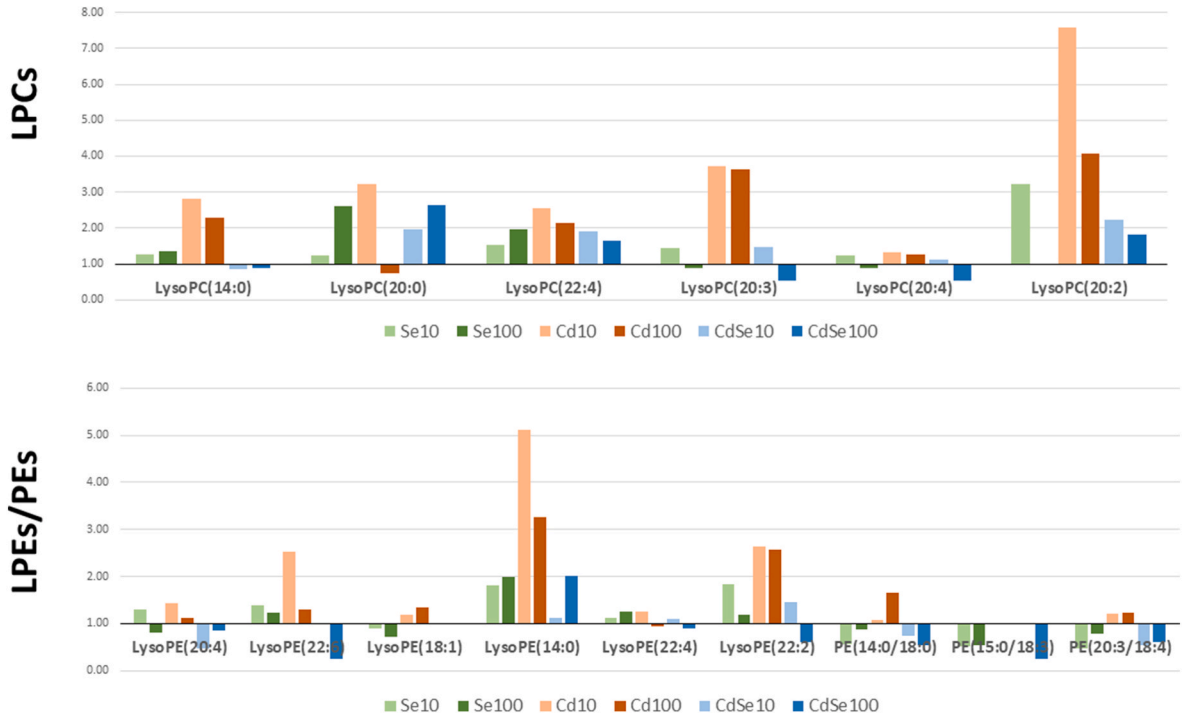

Fig. 6. Bar chart showing fold change values of altered glycerophospholipid compounds in *Scrobicularia plana* under Cd and Se exposure.

enzymes could be responsible of the tissue protection in the digestive gland of *S. plana*. In our study, only total GPx was monitored, and more specific analyses would be necessary to explain the role of this and other molecules on Se prevention against Cd toxicity.

Various authors indicate that Se exposure can have negative effects, including the inhibition of enzymatic activity: it is known that Se can substitute S in the group –SH during the assembly of protein changing their molecular conformation and compromising their functionality (Mayer and Knight, 1994). Additionally, inorganic Se is able to react with thiols generating redox-active intermediates metabolites and consequently producing oxidative stress and related damages to protein, DNA, membranes, etc. (Dörr et al., 2008; Gobi et al., 2018; Gopi et al., 2021). Taking into account these observations, we could attribute the decrease in enzyme activity observed in our work to an adverse effect of Se (both in individual exposure and in mixture with Cd) on the clams. Nevertheless, both the altered activity of antioxidant enzymes and the increase in ROS production should be accompanied by an increase in damage associated with oxidative stress, i.e. lipid peroxidation. This not consistent with our results since we did not observe an increase in LPO levels in clams exposed to Se and, in the case of exposure to the mixture of Cd and Se, the LPO rise was transitory and lower levels with respect to the control were re-established on days 7 and 21. Further studies would be necessary to elucidate beneficial and adverse effects of Se on *S. plana*.

Metabolic profiling revealed changes in the metabolic status of *S. plana* under metal exposure. Consequently, numerous metabolites were identified as biomarkers of Cd exposure. The most important markers were related to AminoacyltRNA biosynthesis, glycerophospholipid metabolism and amino acid metabolism. Next, the significant metabolomic pathways will be commented in the following sections:

- Pathways analysis indicates alterations in glycerophospholipids metabolism in digestive gland of *S. plana* for all exposed groups (Fig. 5). The results show an increase in fold change values of glycerosphopholipid compounds when the clams are exposed to Cd at both concentrations. Moreover, the abundance of these bands is almostre-established to the control clams when clams are exposed to Cd and Se simultaneously. Table S2 shows up-regulation of different classes of phospholipids: (i) *lysophosphatidylcholines* (LPCs), which are degradation products of phosphatidylcholines (PCs) generated by hydrolysis of one fatty acid substituent; and (ii) *lysophosphatidylethanolamines (LPEs),* generated by the degradationof *phosphatidylethanolamines*(PEs). These phospholipids are an important part of plasma membranes, and have energy storage and energy source functions in organisms, since its high content of unsaturated fatty acids (Koivusalo et al., 2001)which, in presence of free radicals, could involve lipid peroxidation that leads to a loss of integrity membrane (Winston and di Giulio, 1991). The alteration in the levels of these metabolitescould indicate a situation of oxidative stress in *S. plana* clams exposed to Cd. Moreover, accumulation of phospholipids could be related to altered hepatic fat metabolism, such as steatosis and phospholipidosis (Breiden and Sandhoff, 2020; Donato and José Gómez-Lechón, 2012). Others authors have demonstrated alteration in lipids content in response to external stressors in invertebrates like *P. clarkii* (Gago-Tinoco et al., 2014), *S. plana* (Rodríguez-Moro et al., 2018), *Mytilus galloprovincialis* mussel (Martin-Diaz et al., 2009) and *Crassostrea gigas* oyster (Wei et al., 2015). Our results shown that membrane deterioration effect is decreased in clams exposed to Cd and Se simultaneously, as can be observed on decreasing fold change values obtained for several metabolites (Fig. 6).
- Table S2 shows variations in the levels of fatty acids and conjugates, such as propionyl-L-carnitine, myristic acid, palmitic acid, stearic acid, 2-methylbutyroylcarnitine and colneleic acid. The results show a general tendency to up-regulate the level of these metabolites when clams are exposed to Cd, especially when they are exposed to 10 μg L^{-1}, although an abrupt increase in the levels of stearic acid is observed when the clams are exposed to 100 μg L^{-1}. Fatty acids are involved in the β-oxidation mechanism, a process in acetyl-CoA is generated from fatty acids, obtaining ATP as final product. The increased levels of fatty acids found in clams under Cd exposure could suggest a situation of hyperlipidemia, which is in accordance with the up-regulated levels of glycerophospholipids. These findings have been previously demonstrated by other authors in marine

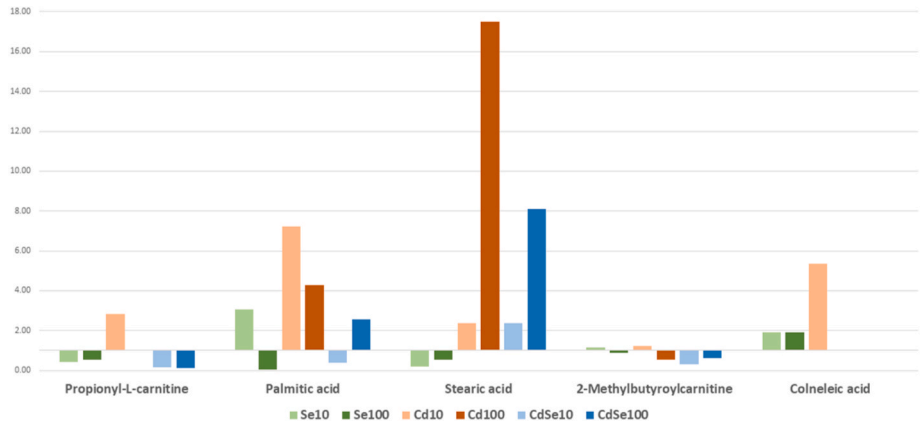

Fig. 7. Bar chart showing fold change values of altered fatty acids and conjugates compounds in *Scrobicularia plana* under Cd and Se exposure.

organisms (Martin-Diaz et al., 2009). When clams are exposed to Cd and Se simultaneously, this effect declines, as can be observed in Fig. 7, where the fold change values of these metabolites are partiallyameliorated when the clams are exposed to Cd together with Se.

- Phosphoric acid was found altered in the analysis by GC-MS. The higher level of phosphoric acid in clams exposed to higher concentration of Cd, could suggest that oxidative phosphorylation mechanism is affected by Cd. Table S2 shows that phosphoric acid present different behavior in the group of clams exposed to Cd or Cd and Se simultaneously when both groups are compared versus the control group. The administration of Se ameliorates the effect of Cd exposure, probably related to an antagonistic interaction between Cd and Se.
- Glutamine is involved in the synthesis of glutamate which participates in mechanisms to fight against the oxidative stress. Increased levels of glutamine have been found in other studies with xenobiotics in aquatic organisms, as *M. galloprovincialis* (Cappello et al., 2013; Jones et al., 2008) or mammals as *Mus musculus* under Cd exposure (García-Sevillano et al., 2014).
- Finally, results shown changes in taurine levels under Cd exposure (Table S2). It has been demonstrated that this metaboliteperforms important roles in several biological functions highlighting their role as stabilizer of cell membranes and a helper in the transport of several cations (Chesney et al., 1990; Miller and Steinberg, 1979). Besides, this amino acid has an important antioxidant activity since able to clean toxins removing free radicals and can reduce the generation of final products of lipid peroxidation (Balkan et al., 2002; Giriş et al., 2008; Ma et al., 2010). These findings have been previously observed in bivalves by other authors, like *M. galloprovincialis* under Hg exposure (Cappello et al., 2013) and Ni (Jones et al., 2008) or *R. Philippinarum* exposed to benzo(a)pyrene (Zhang et al., 2011) and Hg or crayfish *P. clarkii* under environmental pollution (Osuna-Jiménez et al., 2014). These increased levels of taurine are reversed in clams exposed to Se together with Cd.

5. Conclusions

The combination of untargeted metabolomics, oxidative stress and bioaccumulation of metals allowed to delve into the interactions between Se and Cd exposure in bivalves using *S. plana* as model organism. Our results showed that Se modulated Cd bioaccumulation in the digestive gland of clams and ameliorated the oxidative stress. Also, there were up to 53 metabolites altered after Cd exposure at different concentration levels, being the most altered pathways: AminoacyltRNA biosynthesis, glycerophospholipid metabolism and amino acid metabolism. In some cases, the metabolic alterations induced by Cd exposure were ameliorated by Se co-administration, demonstrating the antagonistic action of the later. *S. plana* is an excellentliving organism to be used in environmental contamination assessments, especially those related with environmental metabolomics and metallomics. The combination of powerful analytical techniques such as organic (UHPLC-QTOF and GC-MS) and inorganic mass spectrometry (ICP-MS) allowed a comprehensive study about the antagonism between Cd and Se in bivalves and understand the mechanisms involved in the responses at cellular level.

Credit author statement

Chiara Trombini: Conceptualization, Methodology, Formal analysis, Investigation, Writing, Gema Rodriguez-Moro: Conceptualization, Methodology, Formal analysis, Writing, Sara Ramírez-Acosta: Methodology, Formal analysis, Investigation, José Luis Gómez-Ariza: Conceptualization, Writing-Reviewing and Editing, Julián Blasco: Conceptualization, Writing-Reviewing and Editing, Tamara García-Barrera: Conceptualization, Writing-Reviewing and Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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